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The mouse hepatitis virus (MHV) spike glycoprotein mediates attachment of the virus to the MHV receptor, the murine biliary glycoprotein (BGP) carcinoembryonic antigen. Monoclonal antibody CC1 directed against BGP specifically inhibited infection of DBT, Sac-, GT1-7, and OBL21 cells by wild-type MHV-4 and the neuron-adapted variant OBLV60. Binding to this receptor was necessary to establish infection by cell-free MHV; however, the presence of BGP was not required for infection by cell-associated virus. Cell-associated infection induced syncytium formation on Vero and BHK cells, which lack murine BGP; this activity was not inhibited by monoclonal antibody CC1. Antibody CC1 also did not prevent syncytium formation on DBT cells, which bear BGP. In infectious center assays, the MHV-4 variant OBLV60, which exhibits acid-dependent fusion, spread to cells lacking BGP only when exposed to acidic media. Therefore, spike-mediated fusion was required for BGP-independent spread of MHV infection. Furthermore, BGP-independent, cell-associated spread of MHV-4 was prevented by monoclonal antibodies 5A13.5 and 5B19.2 directed against the spike glycoprotein, but not by other neutralizing and nonneutralizing anti-spike antibodies. Expression of spike glycoprotein by recombinant vaccinia virus resulted in fusion of BGP-negative cells; monoclonal antibodies 5A13.5 and 5B19.2 strongly inhibited spike-mediated fusion in this assay.

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## INTRODUCTION

Virus infection of cells is initiated through the interaction of one or more virion components with receptor molecules on the cell surface. Cellular receptors have been identified for several viruses and the specificity and affinity of the virus–receptor interaction can dictate the course of infection *in vivo*. The tissue distribution and availability of receptors are primary determinants of viral tropism and influence viral pathogenesis. Subsequent to receptor binding, viruses enter the cell through fusion either at the plasma membrane or in endosomal vesicles. Internalization of virus may also be facilitated by additional low or high affinity interactions between virion proteins and cell surface molecules (Fuller and Spear, 1987; Weiss, 1992; Wickham *et al.*, 1993).

The coronaviruses share in common a virion structure marked by distinctive peplomers that extend 200 Å from the surface of the virion (Sturman *et al.*, 1980). The peplomer comprises a homotrimer of spike (S) glycoproteins (Delmas and Laude, 1990) and mediates both virus attachment and membrane fusion. Glycoprotein S is a 180-kDa class I integral membrane protein that is post-translationally cleaved into two 90-kDa fragments called S1 and S2 (Sturman *et al.*, 1985). The amino-terminal S1 portion forms the globular head of the peplomer and

likely contains the receptor-binding domain. The carboxy-terminal S2 portion of S is anchored in the viral membrane by a hydrophobic transmembrane domain. Heptad repeat sequences in S2 are thought to fold into  $\alpha$ -helices that associate with one another in a coiled coil, forming the stalk of the oligomeric peplomer (de Groot *et al.*, 1987). In contrast to the fusion proteins of several other enveloped RNA viruses, cleavage of the murine coronavirus spike glycoprotein does not unmask an apparent N-terminal hydrophobic fusion peptide. The S2 heptad repeat region has, however, been shown to contain residues that influence the pH dependence of fusion (Gallagher *et al.*, 1991).

The receptors for mouse hepatitis virus (MHV) are members of the murine biliary glycoprotein family (BGP), which are carcinoembryonic antigens (CEA) in the immunoglobulin superfamily (Williams *et al.*, 1990, 1991). Two murine *bgp* genes have been characterized, *bgp1* and *bgp2* (Nedellec *et al.*, 1994). Through stable or transient expression of transfected cDNA, four isoforms of BGP1 and one isoform of BGP2 have been shown to render resistant cells susceptible to infection by MHV (Dveksler *et al.*, 1991, 1993; Yokomori and Lai, 1992a; Nedellec *et al.*, 1994); however, in some cases an additional cellular factor may be necessary for viral entry (Yokomori and Lai, 1992b; Yokomori *et al.*, 1993). The N-terminal domain of BGP, which displays additional polymorphism due to allelic variation, functions in virus binding (Dveksler *et al.*, 1993). Monoclonal antibody CC1, directed against the N-terminal domain, has been shown to block virion

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attachment, and thereby inhibit infection of cultured fibroblasts (Williams *et al.*, 1990; Dveksler *et al.*, 1991, 1993). A CEA molecule in the pregnancy-specific glycoprotein subgroup can also serve as a receptor for some strains of MHV, but not for the neurotropic MHV-4 (JHM) strain (Chen *et al.*, 1995).

It has been demonstrated previously that the presence of BGP (formerly called CEA-related MHV receptor) is not required for cell-to-cell spread of MHV-4 infection (Gallagher *et al.*, 1992). Cells lacking BGP were productively infected through fusion into virus-induced syncytia. Cell fusion was shown to be mediated by the spike glycoprotein. Specifically, MHV syncytia developed on monolayers of BHK and RK13 cells when these cells were cocultured with small numbers of monodispersed MHV-infected DBT cells. BHK cells are resistant to MHV infection, but BHK cells transfected with BGP supported MHV infection (Dveksler *et al.*, 1991), indicating that BHK cells possess all of the components required for MHV replication except the receptor. The cell-associated spread of infection to the BGP-negative BHK and RK13 cells led to the production of 250 and 21 times more infectious virus, respectively, than duplicate cultures without a target cell monolayer (Gallagher *et al.*, 1992). Therefore, through cell-to-cell spread of infection, MHV can bypass the receptor-binding event normally required for extracellular infection.

In this report, we examined the receptor utilization by the neurotropic strain MHV-4 and a neuron-adapted variant, OBLV60, in cultured cells of neuronal and nonneuronal origin. We demonstrate that although anti-BGP antibody CC1 prevents MHV-4 and OBLV60 infection of several cell types, it does not block cell-associated spread of infection. This finding supports our contention that the cell-associated spread of MHV does not require the presence of BGP. Further, we investigated the role of spike-mediated fusion in BGP-independent, cell-associated MHV infection and found that this alternate mechanism of spread requires spike-mediated fusion activity. We used vaccinia-based expression of spike glycoprotein to assay fusion activity that is independent of receptor interaction and demonstrate that monoclonal antibodies which inhibit fusion also prevent BGP-independent, cell-associated spread of MHV infection.

## MATERIALS AND METHODS

### Cells

DBT murine astrocytoma (Hirano *et al.*, 1978) and Sac-Moloney sarcoma (Weiland *et al.*, 1978) cell lines were maintained in DMEM with 8% calf serum (Hyclone). BHK-21 cells (baby hamster kidney) were maintained in DMEM/8% calf serum with 0.1× TPB and 0.5% glucose. Vero E6 cells (African green monkey kidney) were maintained in MEM/10% fetal bovine serum (Irvine Scientific) with 0.25% NaHCO<sub>3</sub>. OBL21, a retrovirus-transformed neuronal cell line derived from the olfactory bulb of new-

born CD1 mice (Ryder *et al.*, 1990), and GT1-7, a murine hypothalamic neuronal cell line (Mellon *et al.*, 1990), were maintained in DMEM/10% fetal bovine serum. All media solutions were supplemented with 25 mM HEPES and 2 mM L-glutamine.

### Virus

The MHV-4 strain of virus was originally obtained from M. Haspel (Haspel *et al.*, 1978) and is routinely passaged in Sac- cells. The OBLV60 variant was isolated on Day 60 of a persistent MHV-4 infection established in OBL21a cells (Gallagher *et al.*, 1991).

### Antibodies

The generation and characterization of monoclonal antibodies against MHV-4 and the production of ascites fluid has been previously described (Collins *et al.*, 1982; Buchmeier *et al.*, 1984). The specificity of the monoclonal antibodies against the nucleocapsid (N), membrane (M), or S proteins are as follows: 4B6.2 (N), 5A5.2 (M), 5B11.2 (M), 5A13.5 (S), 5B19.2 (S), 4B11.6 (S), 5B93.3 (S), 5B21.5 (S), 5B207.7 (S), and 5B216.8 (S). Monoclonal antibody 1-1.3 recognizing the lymphocytic choriomeningitis virus (LCMV) nucleocapsid protein was used as a control antibody against an unrelated antigen (Buchmeier *et al.*, 1981). Anti-BGP antibody CC1 hybridoma supernatant was generously provided by Kathryn Holmes.

### Plaque assays

Viral titers were determined by plaque assay in which DBT cell monolayers in six-well culture plates were inoculated with serial log or half-log dilutions of virus. After a 1-hr absorption period, the inoculum was removed and replaced with overlay medium (DMEM, 1% calf serum, 0.5% SeaKem agarose). After plaque development, the monolayers were fixed with ~5% paraformaldehyde, then stained with 0.1% crystal violet after removal of the agarose overlay.

To quantitate the capacity for anti-BGP antibody CC1 to inhibit MHV-4 infection, plaque reduction assays were performed as follows. DBT or Sac- cell monolayers in six-well culture plates were incubated with either no antibody or half-log dilutions of antibody CC1 for 1 hr before and 2 hr after infection with a standardized inoculum of MHV-4 (150 PFU). The inoculum was then removed and replaced with overlay medium. Fixation and staining were performed as described above. The ability of antibody CC1 to inhibit infection with MHV-4 or OBLV60 was also assessed microscopically. Confluent monolayers of GT1-7, OBL21, DBT, or Sac- cells were pretreated for 1 hr at 37° with antibody CC1 at twice the dilution indicated, before addition of an equal volume of virus. After 16 to 20 hr of incubation at 37°, cells were fixed and processed for immunofluorescence.

The neutralizing titer of ascites fluid containing monoclonal antibody against MHV-4 was quantitated by

plaque reduction assay as follows. Ascites fluids were serially diluted in DMEM and mixed with an equal volume (0.2 ml) of a standardized inoculum of MHV-4 (100 PFU). The virus-antibody mixtures were incubated at 4° for 1 hr, then used to inoculate DBT cell monolayers in six-well culture plates. Following a 1-hr absorption at 37°, the inoculum was removed and replaced with overlay medium. Fixation and staining were performed as described above.

The capacity of ascites fluid to inhibit cell-cell fusion was quantitated by plaque reduction assay. DBT cell monolayers were incubated with a standardized inoculum of MHV-4 (100 PFU) for 1 hr at 37°. The inoculum was then removed and replaced with overlay medium containing ascites fluid at serial dilutions. Fixation and staining were performed as described above.

### Indirect immunofluorescence

Cells that were grown on glass coverslips or Nunc Lab-Tek chamber slides were fixed with 10% zinc-formalin for 5 min at room temperature, permeabilized with 2% NP-40 for 10 min, then blocked with 5% normal goat serum (Vector Laboratories) for 15 min or more. The cells were bathed for 1 hr or more with a cocktail of anti-MHV antibodies (4B6.2, 5A5.2, and 5B19.2 or 5A13.5, recognizing nucleocapsid, membrane, and spike proteins, respectively) at a 1:100 dilution in phosphate-buffered saline (PBS) containing 1% normal goat serum, then incubated for 1 hr with a TRITC-conjugated, sheep anti-mouse antibody (Accurate), also diluted in PBS containing 1% normal goat serum. Cells were mounted in 90% glycerol containing 25 mg/ml 1,4-diazobicyclo-2,2,2-octane in PBS and visualized by fluorescence microscopy.

### Infectious center assay

Adherent DBT cells were removed from tissue culture flasks by treatment with Nonenzymatic Cell-Dissociation Solution (Sigma) and infected with MHV-4 or OBLV60 in suspension at a multiplicity of 5 PFU per cell. The cells were incubated at 37° for 1 hr, then washed extensively in cold PBS or OptiMem serum-free medium (Gibco). The infected cells were serially diluted in OptiMem media containing monoclonal antibody CC1 (1:100 dilution), then seeded onto untreated glass coverslips or coverslips covered with confluent BHK, Vero, or DBT cell monolayers. All target cell monolayers had been pre-treated with monoclonal antibody CC1 (1:50 dilution) for 1 hr; a 1:100 dilution of antibody CC1 was present throughout the course of the experiment. For specified experiments, anti-spike monoclonal antibody 5A13.5, 5B19.2, 4B11.6, 5B93.3, or 5B21.5 was also included at a 1:100 dilution. At 20 hr postinfection, the cells were fixed and infected cells were visualized by indirect immunofluorescence.

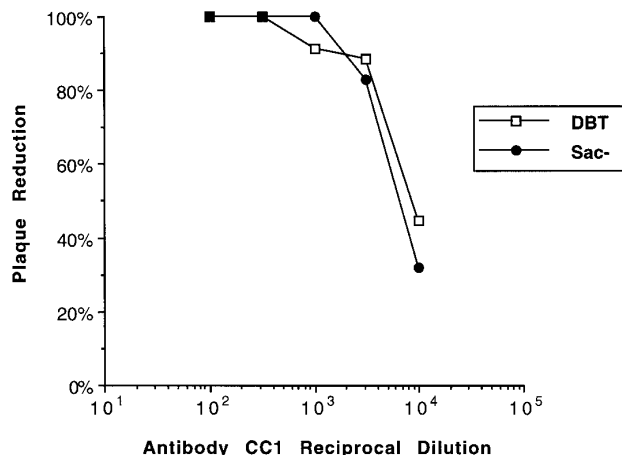


FIG. 1. Inhibition of MHV-4 infection of DBT and Sac- cells with anti-BGP monoclonal antibody CC1. DBT or Sac- cell monolayers were incubated with the indicated dilutions of antibody CC1 for 1 hr before and 2 hr after infection with MHV-4. Culture media were then removed and replaced with agarose overlay media. Following incubation at 37° for 2 days, cells were fixed with paraformaldehyde, then stained with crystal violet.

### Expression of recombinant S glycoprotein

BHK or DBT cell monolayers grown on glass coverslips were co-infected with the recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase, vTF7.3 (Fuerst *et al.*, 1986), and a recombinant vaccinia virus that has the MHV-4 spike cDNA under the control of the T7 RNA polymerase promoter (vv-S). The spike vaccinia recombinant had been derived through homologous recombination between vaccinia DNA and MHV-4 spike cDNA inserted into the pTM-1 vector (T. M. Gallagher and M. J. Buchmeier, unpublished data, 1994). Expression of recombinant spike glycoprotein was detected by indirect immunofluorescence. For specified experiments, anti-spike monoclonal antibodies 5A13.5, 5B19.2, 4B11.6, 5B93.3, 5B21.5, 5B207.7, or 5B216.8 or control antibodies directed against BGP (CC1), the membrane protein (5B11.5), nucleocapsid protein (4B6.2), or the LCMV nucleocapsid protein (1-1.3) were included at a 1:100 or 1:500 dilution.

## RESULTS

### Anti-BGP antibody CC1 inhibits both MHV-4 and OBLV60 infection

Dveksler *et al.* (1991) have demonstrated that BGP is a functional receptor for MHV strain A59 by inhibiting infection with the anti-BGP monoclonal antibody CC1. To confirm that the neurotropic MHV-4 strain also uses this receptor, we performed receptor-blocking experiments with monoclonal antibody CC1. Figure 1 shows that antibody CC1 led to a reduction of MHV-4 plaque formation assayed on two different murine cell lines. Complete inhibition was measured at dilutions of antibody CC1 less than 10<sup>-2.5</sup>. The 50% reduction in plaque number was

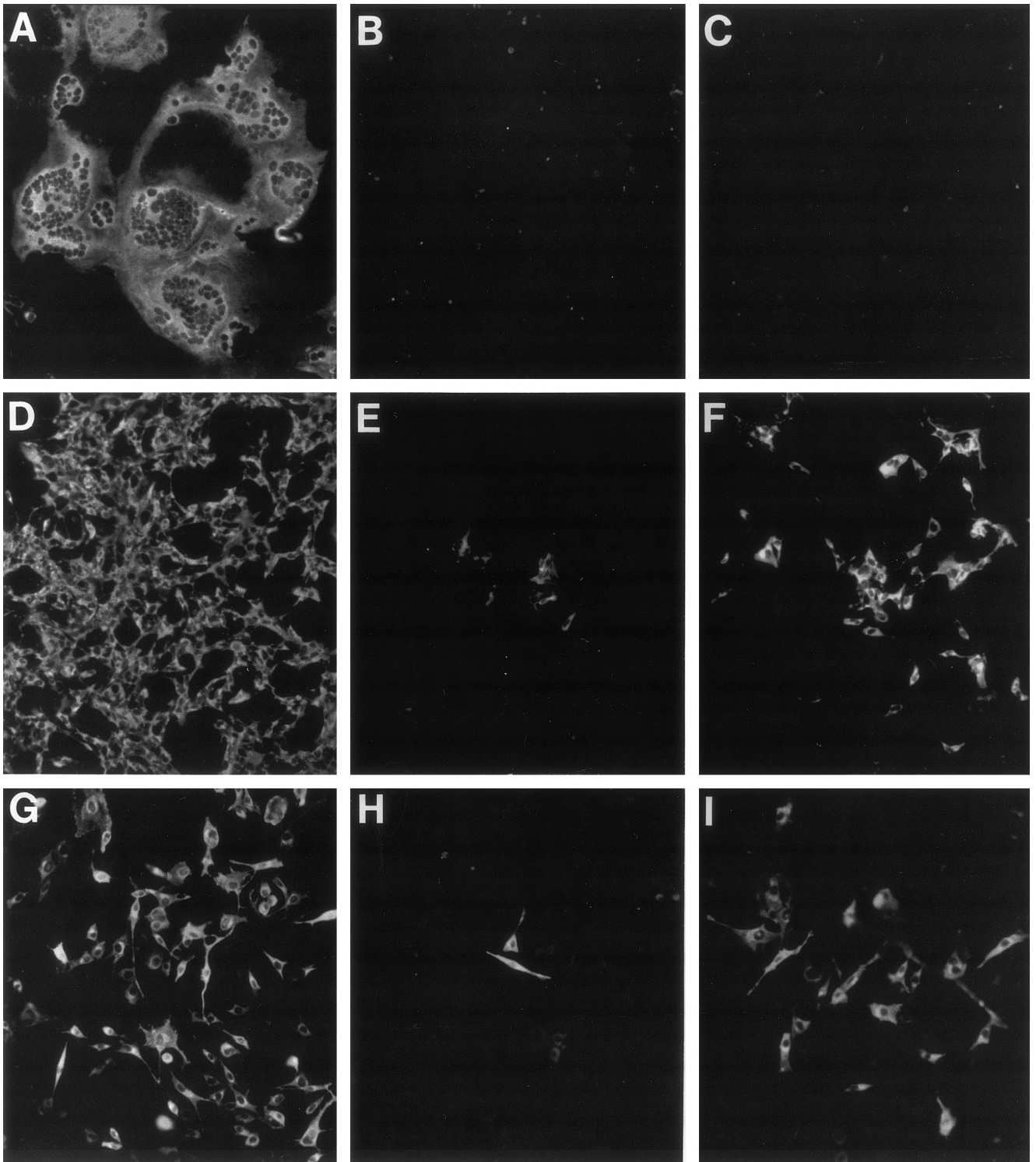
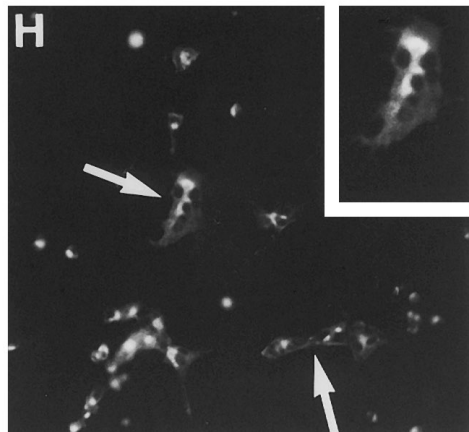
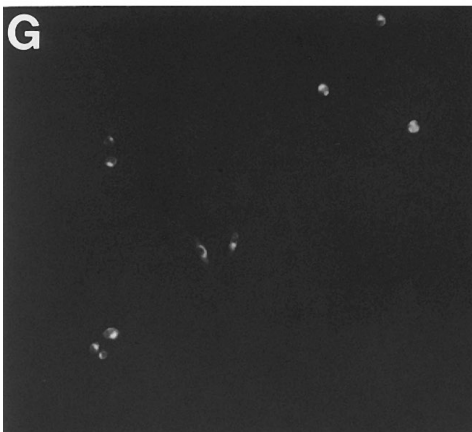
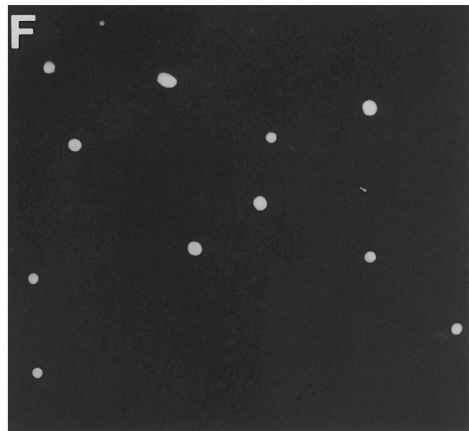
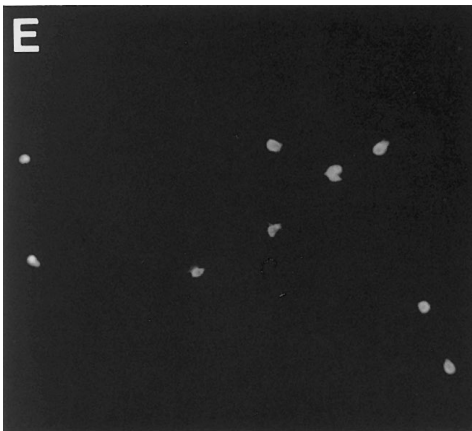
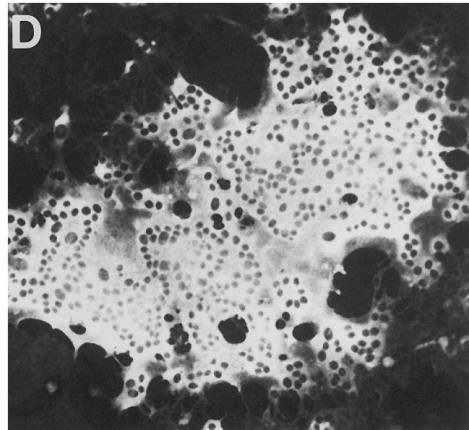
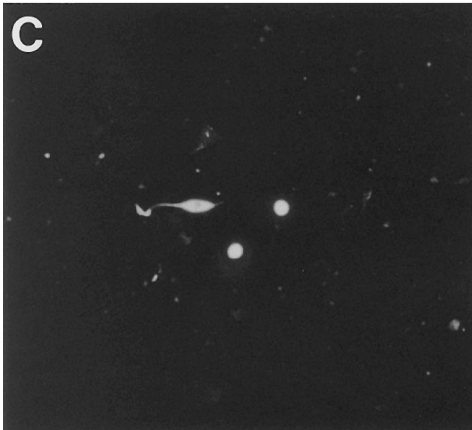
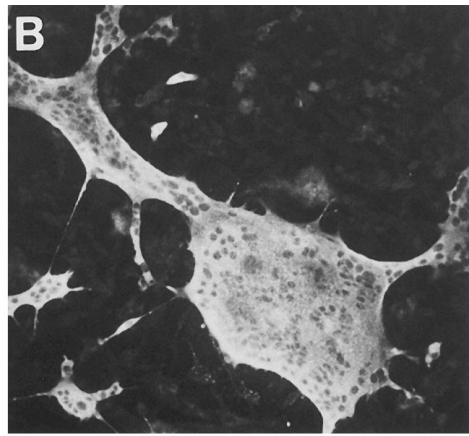
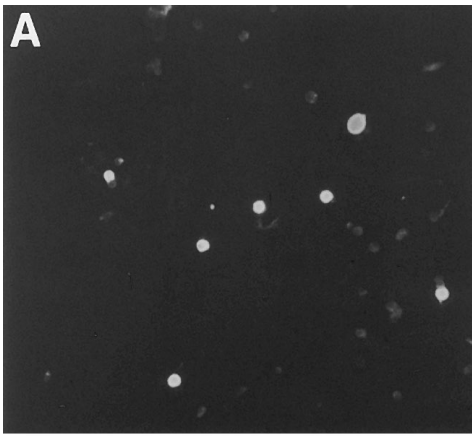


FIG. 2. Inhibition of MHV-4 infection of GT1-7 cells and OBLV60 infection of GT1-7 and OBL21 cells by anti-BGP monoclonal antibody CC1. Cell cultures were either (A, D, G) untreated or pretreated for 1 hr with antibody CC1, which was present at dilutions of (B, E) 1:10, (H) 1:20, or (C, F, I) 1:100 throughout the course of infection. Confluent cell monolayers were infected with MHV-4 (A–C) or OBLV60 (D–I). Following a 20-hr incubation, MHV antigen was detected by indirect immunofluorescence.

greater than  $10^{-3.5}$  for antibody CC1 on both cell lines. The dose-dependent plaque reduction indicates that antibody CC1 blocks the MHV receptor that is utilized by MHV-4 in both DBT and Sac- cells.

The ability of anti-BGP monoclonal antibody CC1 to inhibit infection with MHV-4 or OBLV60 was also assessed microscopically, in which case the extent of infection was visualized following indirect immunofluorescent



staining of viral antigen. MHV infection of the neuronal cell lines GT1-7 and OBL21 does not cause the formation of discernible plaques; however, there is clearly a dose-dependent inhibition of MHV-4 and OBLV60 infection of these cell lines by monoclonal antibody CC1. Representative examples in which confluent monolayers of these cells were inoculated with MHV-4 or OBLV60 with or without monoclonal antibody CC1 pretreatment are shown in Fig. 2. At a  $10^{-2}$  dilution of antibody CC1, in fields of more than  $10^5$  cells, no MHV-4-infected GT1-7 cells were detected (Fig. 2C), and the number of OBLV60-infected GT1-7 (Fig. 2F) or OBL21 (Fig. 2I) cells detected was reduced by more than 50% compared to the corresponding cultures without antibody CC1 (Figs. 2D and 2G). At higher concentration of antibody CC1, only an occasional OBLV60-infected GT1-7 (Fig. 2E) or OBL21 (Fig. 2H) cell was detected. Together, these results demonstrate that infection of both neuronal and nonneuronal cells by cell-free MHV-4 and OBLV60 requires the presence of accessible BGP.

#### Anti-BGP antibody CC1 does not prevent cell-to-cell spread of MHV infection

Cell-associated spread of MHV to cells that lack murine BGP has been demonstrated with an infectious center assay in which infection spread from MHV-infected DBT cells to BGP-negative target cell monolayers (Gallagher *et al.*, 1992). To further test the role of BGP in cell-associated spread of MHV infection, infectious center assays were performed with inclusion of the receptor-blocking monoclonal antibody CC1 in the culture media. Confluent BHK, Vero, and DBT cell monolayers were preincubated with antibody CC1 for 1 hr, then overlaid with a suspension of MHV-4-infected DBT cells. At 20 hr postinfection, infected DBT cells plated without an underlying monolayer were widely dispersed and remained single cells (Figs. 3A and 3C), whereas large syncytia were evident in the cultures with BHK (Fig. 3B), DBT (Fig. 3D), or Vero (data not shown) target cell monolayers. The number of syncytia was approximately equal to the number of infected single cells in the cultures without an underlying monolayer (data not shown), consistent with a cell-to-cell rather than extracellular mode of virus transmission. The presence of syncytia indicates that infection spread from the primary infected DBT cells to the BHK, Vero, and DBT cell monolayers, despite the presence of antibody CC1 in the culture medium. The inability of monoclonal antibody CC1 to prevent infection of target cell monolayers in these infectious center assays further

supports the conclusion that cell-associated spread of MHV can occur without BGP serving as the MHV receptor.

#### Spike-mediated fusion is required for BGP-independent cell-to-cell spread of MHV infection

OBLV60 is a fusion variant of MHV-4 that arose during persistent infection of the OBL21a cell line (Gallagher *et al.*, 1991), a neuronal cell line that is refractory to MHV-induced cell fusion. Due to three amino acid mutations that occurred in the heptad repeat region of S2, the OBLV60 spike glycoprotein requires acid activation in order to mediate membrane fusion (Gallagher *et al.*, 1991). Consequently, infection of fusion-sensitive cells with OBLV60 leads to syncytium formation only in acidic culture medium.

To assess the role of fusion in BGP-independent, cell-associated spread of MHV, we tested whether infection spreads in a BGP-independent manner from cells infected with the OBLV60 acid-dependent fusion variant. An infectious center assay was performed in which OBLV60-infected DBT cells were seeded with (Figs. 3F and 3H) and without (Figs. 3E and 3G) an underlying monolayer of confluent BGP-negative BHK or Vero target cells. As above, monoclonal antibody CC1 was present throughout the course of the experiment. In neutral pH culture medium, OBLV60 infection remained isolated to the single cells of the overlay and failed to spread to receptor-negative target cells (Fig. 3F). However, when exposed to acidic (pH 5.0) medium for 1 hr, small syncytia developed as a result of one round of fusion between the OBLV60-infected cells and adjacent BGP-negative cells; return to neutral pH prevented the further spread of infection. The ability of the OBLV60 fusion variant to spread to BGP-negative cells only at acidic pH demonstrates that spike glycoprotein fusion capability is required for BGP-independent, cell-associated spread of MHV infection.

#### BGP-independent, cell-associated spread of MHV-4 is prevented by fusion-inhibiting anti-spike monoclonal antibodies

We have previously reported the generation and characterization of a panel of neutralizing and nonneutralizing monoclonal antibodies directed against the MHV-4 spike glycoprotein (Collins *et al.*, 1982; Buchmeier *et al.*, 1984). The results of plaque reduction assays quantitating the neutralization and fusion inhibition titers for monoclonal antibody ascites preparations used in this study are

**FIG. 3.** Cell-associated spread of MHV infection despite the presence of anti-BGP antibody CC1 and BGP-independent spread of cell-associated OBLV60 at acidic pH. DBT cells were infected with (A–D) MHV-4 or (E–H) OBLV60 in suspension for 1 hr, then washed extensively to remove unbound virus. Aliquots of infected DBT cells were overlaid onto blank coverslips (A, C, E, G), confluent BGP-negative BHK cell (B, F), or Vero cell monolayers (H) or confluent BGP-positive DBT cell monolayers (D). Following an 18-hr incubation, the cells shown in (H) were exposed to MES-buffered DMEM (pH 5.0) for 1 hr. Small OBLV60-induced syncytia that appeared after acid exposure are marked with arrows and shown enlarged in the inset. Antibody CC1 was present at 1:100 dilution in all cultures throughout the experiment. MHV antigen was detected by indirect immunofluorescence at 20 hr postinfection.

shown in Fig. 4. The plaque reduction values measured for antibody dilutions of 1:100 are listed in Table 1. Monoclonal antibodies 5B19.2, 5A13.5, and 4B11.6 were strongly neutralizing, reducing plaque formation 100% at a 1:100 dilution. Antibodies 5B93.3 (78.4%) and 5B21.5 (48.6%) were moderately and weakly neutralizing, respectively; antibody 5B207.7 (4.4%) was essentially nonneutralizing (Fig. 4A). Only antibodies 5A13.5 (90%) and 5B19.2 (81.6%) strongly inhibited cell fusion. Monoclonal antibodies 4B11.6 (58.8%) and 5B21.5 (36.7%) partially inhibited fusion, whereas antibodies 5B93.3 (9.4%) and 5B207.7 (0%) did not prevent fusion (Fig. 4B). The plaques were significantly smaller than normal in the assays with antibody 5A13.5 at all dilutions, or antibodies 5B19.2 or 4B11.6 at dilutions of  $10^{-2}$ ,  $10^{-2.5}$ , and  $10^{-3}$ .

To examine the structural and functional features of the spike glycoprotein that are important for receptor-

TABLE 1

Functional Activities of Anti-Spike Monoclonal Antibodies

Monoclonal antibody	Neutralization <sup>a</sup>	PRA fusion inhibition <sup>a</sup>	vv-S fusion inhibition <sup>a</sup>	BGP-IS inhibition <sup>a</sup>	<i>In vivo</i> protection <sup>b</sup>
5A13.5	100%	90.0% <sup>c</sup>	+	+	+
5B19.2	100%	81.6% <sup>c</sup>	+	+	+
4B11.6	100%	58.8% <sup>c</sup>	—	+/-	—
5B93.3	78.4%	9.4%	—	—	—
B521.5	48.6%	36.7%	—	—	—
B5207.7	4.4%	0.0%	—	ND	—

<sup>a</sup> Antibody dilutions 1:100; PRA, plaque-reduction assay; vv-S, vaccinia virus-expressed spike glycoprotein; BGP-IS, BGP-independent spread; ND, not determined.

<sup>b</sup> Buchmeier *et al.*, 1984.

<sup>c</sup> Small plaque morphology.

independent syncytia formation, we tested the ability of anti-spike monoclonal antibodies to interfere with BGP-independent spread of MHV-4 infection in an infectious center assay. MHV-4-infected DBT cells were bathed in media containing individual anti-spike monoclonal antibodies (1:100 dilution) before being seeded onto confluent BHK cell monolayers. Both anti-spike monoclonal antibodies and anti-receptor antibody CC1 were present at a 1:100 dilution throughout the course of the experiment. Two antibodies, 5A13.5 and 5B19.2, routinely prevented MHV-induced syncytia formation; infectious centers remained mononuclear (Figs. 5B and 5C). Monoclonal antibody 4B11.6 inhibited, but did not consistently prevent BGP-independent spread of MHV-4. In some experiments, a few small syncytia were detected in the presence of antibody 4B11.6; however, most infectious centers remained mononuclear (Fig. 5D). Monoclonal antibodies 5B93.3 and 5B21.5 had no apparent effect on BGP-independent spread of MHV-4. In the presence of antibodies 5B93.3 (Fig. 5E) or 5B21.5 (Fig. 5F), cell-cell fusion led to the formation of syncytia that were approximately the same size at the syncytia that developed in the absence of anti-spike monoclonal antibodies (Fig. 5A) and no mononuclear infectious centers were detected.

**Inhibition of recombinant spike-mediated fusion by anti-spike monoclonal antibodies**

MHV-induced fusion activity can be mediated by the spike glycoprotein alone; no other viral components are required (Pfleiderer *et al.*, 1990; Gallagher *et al.*, 1991). We (Gallagher *et al.*, 1992) and others (Taguchi *et al.*, 1992) have demonstrated that expression of recombinant MHV spike glycoprotein leads to syncytia formation on both BGP-positive and BGP-negative cells. Furthermore, there is no qualitative difference in the degree of fusion induced by vaccinia virus-expressed recombinant spike glycoprotein on DBT (BGP-positive) or BHK (BGP-negative) cells when antibody CC1 is included in the culture medium (data not

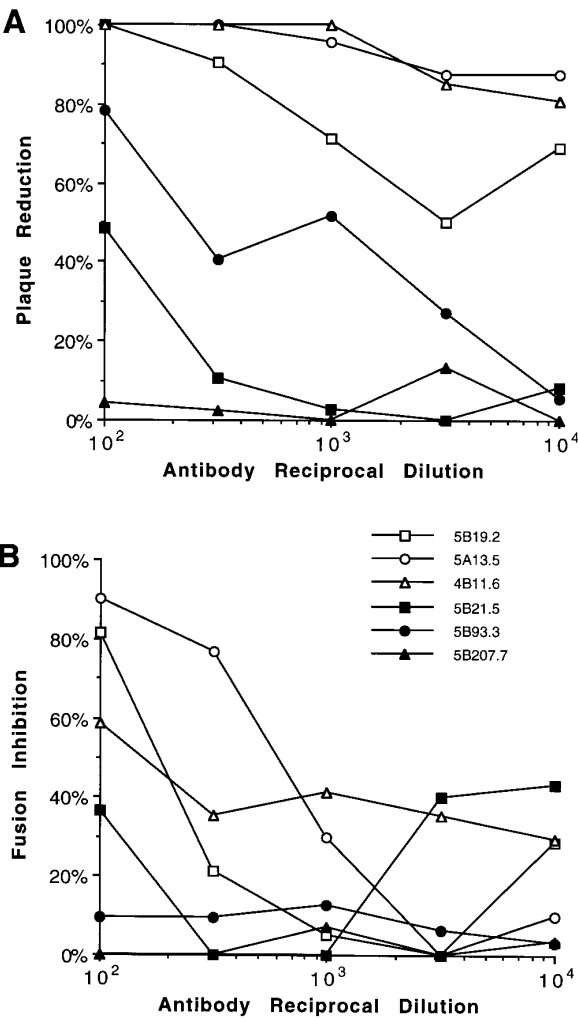
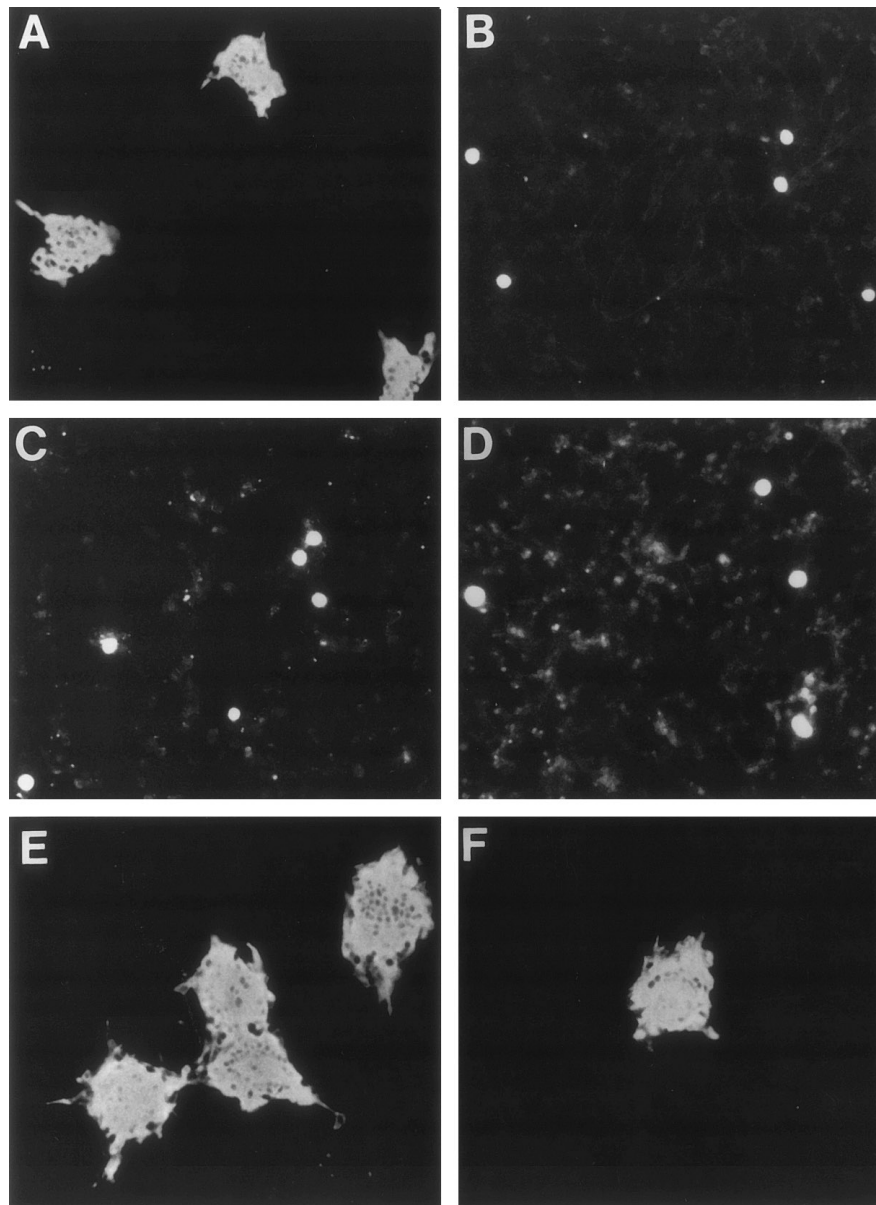


FIG. 4. MHV-4 neutralization and inhibition of syncytia formation by anti-spike monoclonal antibodies. (A) Neutralization plaque-reduction assay. MHV-4 was incubated with the indicated dilutions of ascites fluid for 1 hr prior to infection of DBT cells. (B) Fusion inhibition plaque-reduction assay. DBT cell were infected with MHV-4 for 1 hr, then overlaid with overlay medium containing ascites fluid at the indicated dilutions.





**FIG. 5.** Prevention of BGP-independent, cell-associated spread of MHV-4 by fusion-inhibiting anti-spike monoclonal antibodies 5A13.5 and 5B19.2. MHV-4-infected DBT cells in suspension were treated with anti-spike monoclonal antibodies (1:100) and overlaid onto confluent BHK cell monolayers in the presence of antibody CC1. (A) No anti-spike antibody, (B) 5A13.5, (C) 5B19.2, (D) 4B11.6, (E) 5B93.3, and (F) 5B21.5. Viral antigen was detected by indirect immunofluorescence.

shown). These results demonstrate that expression of recombinant spike glycoprotein in BGP-negative cells provides an assay for fusion that is independent of receptor interactions. Here, we used this assay to evaluate the capacity of anti-spike monoclonal antibodies to specifically block spike-mediated fusion.

BHK cells were co-infected with recombinant vaccinia viruses expressing T7 RNA polymerase (Fuerst *et al.*, 1986) and the MHV-4 spike glycoprotein (w-S) in the presence of 1:100 or 1:500 dilutions of the following antibodies: 5A13.5, 5B19.2, 4B11.6, 5B21.5, 5B93.3, 5B207.7, 5B216.8, 5B11.5 (anti-M), 4B6.2 (anti-N), CC1 (anti-BGP), and 1-1.3 (anti-LCMV NP). Recombinant spike glycoprotein expression caused syncytia formation in all cases tested except where

antibodies 5A13.5 or 5B19.2 were present; antibodies 5A13.5 or 5B19.2 inhibited spike-mediated fusion in this assay at both 1:100 and 1:500 dilutions. Representative examples of w-S-induced syncytia and fusion inhibition by antibody 5A13.5 are shown in Fig. 6.

In summary, BGP-independent spread of MHV-4 infection was inhibited by anti-spike monoclonal antibodies that strongly inhibit MHV-induced syncytium formation and spike-mediated cell fusion, but not by neutralizing or nonneutralizing antibodies that do not inhibit fusion.

## DISCUSSION

We have demonstrated that cell-associated MHV can bypass the requirement for binding to its primary recep-



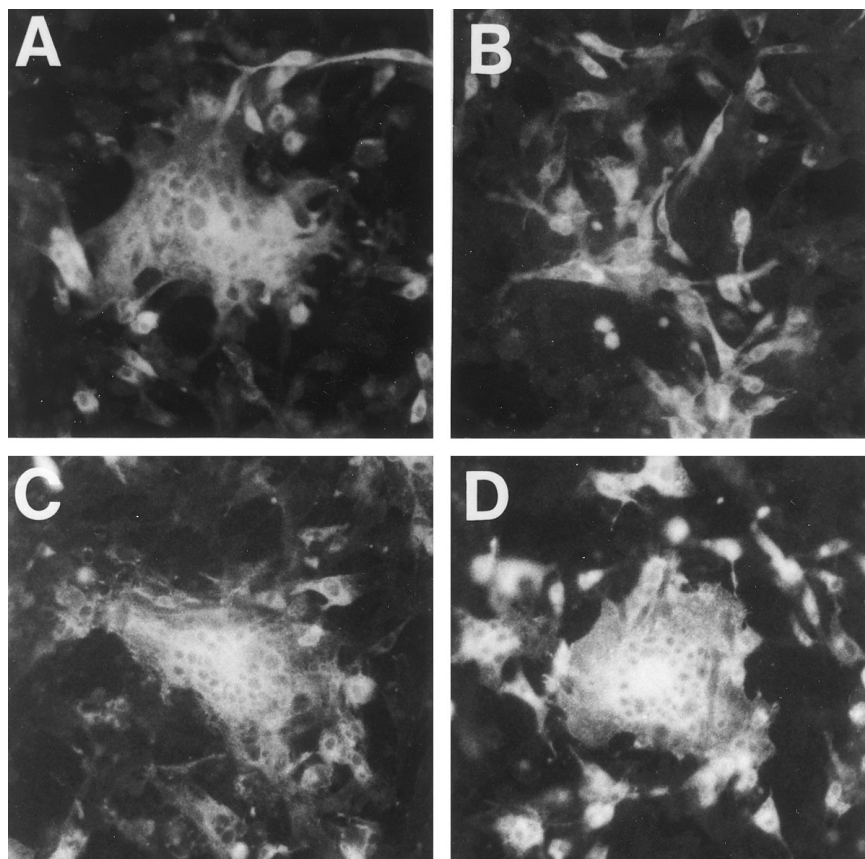


FIG. 6. Inhibition of recombinant spike-mediated fusion by anti-spike monoclonal antibody 5A13.5. Recombinant MHV-4 spike glycoprotein was expressed by a vaccinia virus-based system with or without the presence anti-spike monoclonal antibodies. (A) No anti-spike antibody, (B) 5A13.5 (1:500), (C) 5B93.3 (1:100), and (D) 5B21.5 (1:100). Spike antigen was detected with indirect immunofluorescence.

tor, BGP, and thereby spread to cells that are resistant to infection by cell-free MHV. Anti-BGP antibody CC1, which blocked infection by MHV virions, failed to prevent cell-associated spread of MHV to BGP-negative or BGP-positive cells. The capacity for BGP-independent, cell-associated spread may permit greater dissemination of infection *in vivo* and consequently may augment virulence. We previously demonstrated that the MHV-4 strain, which is highly neurovirulent, spread to BGP-negative cells in culture more efficiently than the less neurovirulent A59 strain (Gallagher *et al.*, 1992); however, these two strains do differ significantly, particularly in the S1 domain of the spike glycoprotein, and therefore this observation does not conclusively demonstrate a correlation between neurovirulence and capacity for receptor-independent spread. In an *in vivo* prophylaxis experiment, anti-BGP monoclonal antibody CC1 failed to fully protect infected mice against encephalitis (Smith *et al.*, 1991). The inability of anti-BGP antibody to completely prevent spread of infection to the brain is not surprising if a BGP-independent component of MHV spread operates *in vivo*. This alternate mechanism of spread may also explain how MHV, which does not infect primate fibroblasts in culture (Compton *et al.*, 1992), did infect primates inoculated with a suspension of infected murine cells or brain homogenate (Murray *et al.*, 1992).

We further demonstrated that BGP-independent, cell-associated spread of MHV requires viral fusion capability; the acid-dependent MHV fusion variant OBLV60 spread through a BGP-independent mechanism only under conditions permissive for fusion. Viral fusogenicity is a function of the spike glycoprotein and expression of recombinant spike glycoprotein alone is sufficient to induce syncytia formation. Recombinant MHV-4 spike glycoprotein fused BGP-negative cells and was unaffected by anti-BGP antibody CC1; these results segregate the receptor-binding and fusion activities of the MHV spike glycoprotein. Theoretically, dissemination of MHV infection through a BGP-independent, cell-associated mechanism *in vivo* provides the potential for spread of infection to cells that lack BGP or despite the presence of neutralizing antibodies. Only anti-spike monoclonal antibodies 5A13.5 and 5B19.2 prevented BGP-independent, cell-associated spread of MHV infection. These antibodies strongly inhibited spike-mediated fusion in MHV plaque reduction assays and vaccinia-based expression experiments. Interestingly, *in vivo* administration of these same two antibodies has been shown to attenuate MHV infection and protect against fatal encephalitis in mice (Buchmeier *et al.*, 1984). A summary of the functional features of several anti-spike monoclonal antibodies is presented in Table 1.

Information about the structural domains of the spike glycoprotein recognized by the antibodies used in this study is limited. Monoclonal antibody 5B19.2 recognizes a linear epitope (Luytjes *et al.*, 1989) located in an immunodominant neutralization domain in a conserved region of S2 (Daniel *et al.*, 1993). Antibodies 5A13.5 and 4B11.6 recognize topographically distinct, conformation-dependent epitopes (Talbot *et al.*, 1984). Neuroattenuated MHV variants V5A13 and V4B11 (Dalziel *et al.*, 1986), which escaped neutralization by antibodies 5A13.5 and 4B11.6, respectively, have deletions in S1 (Parker *et al.*, 1989; Gallagher *et al.*, 1990); however, it is not known whether the monoclonal antibody contact residues lie within the deleted region or whether the deletions affect protein conformation at other sites. Neutralization-resistant variants with point mutations, rather than deletions, have also been isolated (Gallagher *et al.*, 1990); we are currently in the process of determining the nucleotide sequence of these variant spike genes in order to precisely identify the 5A13.5 and 4B11.6 antibody epitopes.

We propose two possible mechanisms for BGP-independent spread of MHV. First, the spike glycoprotein may be binding to an alternate, low-affinity receptor that is inadequate for initiating infection by virions. The presence of spike glycoprotein at high density on infected cell surfaces may allow multivalent interactions with the target cell, thus bypassing the requirement for a high affinity receptor. It has been shown previously that MHV is able to utilize an alternative receptor, the hemagglutinin molecule of influenza virus (Fuller *et al.*, 1985), which is expressed at high levels on the surface of influenza-infected cells. Neuraminidase-treated MHV was unable to infect influenza-infected cells, demonstrating that the sialic acid-binding activity of influenza hemagglutinin mediates MHV infection. Interestingly, influenza hemagglutinin was also shown to be able to serve as a receptor for vesicular stomatitis virus and Semliki Forest virus, both of which require acid activation for membrane fusion. Other reports of viral infection through the use of alternate receptors include the asialoglycoprotein receptor on a liver-derived cell line serving as a receptor for Sendai virus (Markwell *et al.*, 1985), and galactosylceramide serving as a receptor for HIV-1 on CD4-negative neuronal (Bhat *et al.*, 1991; Harouse *et al.*, 1991) and colonic epithelial cell lines (Fantini *et al.*, 1993).

A second possible mechanism for BGP-independent spread is that the spike glycoprotein itself does not bind to receptor molecules on target cells, but rather that through the interaction of cell surface adhesion molecules, adjacent cell membranes are placed in close enough proximity for the spike glycoprotein to initiate cell-cell fusion. We have demonstrated that the extent of BGP-independent spread of MHV varies depending on the type of target cell (Gallagher *et al.*, 1992). The susceptibility of different cell types of BGP-independent infection might be a function of the nature and extent of cell-cell contact. Additionally, MHV-induced fusion is

known to be affected by the lipid composition of the plasma membrane (Daya *et al.*, 1988; Roos *et al.*, 1990).

Other viruses that can mediate membrane fusion at neutral pH may also have the capacity to spread through receptor-independent mechanisms. Interestingly, the fusogenic transmembrane protein of HIV, gp41, has been reported to induce syncytium formation when expressed in cells normally resistant to infection with HIV virions, suggesting that receptor-independent, cell-associated spread may also occur during HIV infection (Perez *et al.*, 1992). The capability of viruses to bypass binding to primary receptors through, for example, the use of alternate receptors or virus-mediated cell-cell fusion needs to be considered in the design of potential receptor-targeted antiviral agents.

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